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13. ABSTRACT ***(Maximum 200 Words)***

Our overall aim is to identify peptide motifs or molecules that may mediate the specific homing of metastatic tumor cells to bone. Our approaches involves the use of random peptide libraries expressed on the surface of filamentous phage as well as an expression cloning strategy using immortalized bone marrow stromal and endothelial cells to detect the binding of Cos-1 cells transfected with cDNAs from the bone metastatic MDA-MB-231 breast cancer cell line.

Using both these approaches we have successfully identified 10 peptides by in vivo phage display and two novel cDNAs (A3 and A5) by expression cloning and work continues on the characterization of these molecules. In particular by Northern blot analysis the A3 mRNA is expressed in high amount in bone metastatic tumor cell lines, while A5 appears to be overexpressed in breast- and prostate cancer cell lines irrespective of their metastatic potential.

These experimental approaches will lead to the discovery of molecules that may help us uncover the basis mechanisms of bone metastasis by cancer cells which remains today one of fundamental unresolved problems in tumor biology. Furthermore, identification of bone specific homing sequences could enable us to design vectors to be used in gene therapy of genetic diseases effecting bone and/or to block bone metastasis.

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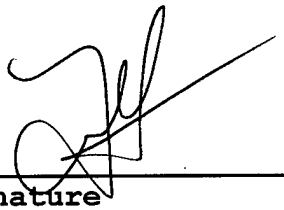

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4. Table of Contents

5. INTRODUCTION

Bone is the most common site of metastasis of breast cancer cells and approximately 70 % of patients with breast cancer have skeletal metastasis by the time of autopsy. Despite the frequent occurrence of bone metastasis and their great consequences to the patients, the mechanisms that favor bone as a site of metastasis of breast cancer cells are not known. There is no information available about the homing molecules or homing receptors that may be participating in this process. We are using two complementary approaches to try to uncover molecules that home specifically to bone. One is a novel approach developed at our institute which makes use of random peptide libraries expressed on the surface of filamentous phage in order to identify peptides that may confer preferential homing properties. The second is a more classical panning strategy, whereby a metastatic breast cancer cell line is used to generate a cDNA library that is then expressed in a mammalian expression system. To aid this approach we have generated a number of immortalized bone marrow stromal and endothelial cell lines and developed a novel binding assay to identify the binding of transfectants onto the bone marrow cell lines. Both of these experimental approaches may lead to uncovering the basic mechanisms of bone metastasis by cancer cells which remain today one of the fundamental unresolved problems in tumor biology. In a more general application, identification of bone specific homing sequences would enable the design of vectors to be used in gene therapy of genetic diseases affecting bone.

6. BODY

The approved tasks for this project include:

1) To generate and screen random peptide libraries in vivo to identify sequence motifs homing specifically to bone.

2) To ascertain if the peptide sequences exist in the context of full length cDNA molecules present on metastatic breast cancer cells

3) To identify and clone the receptors on the surface of bone marrow endothelial cells that are recognized by the homing peptides.

During this second year of support we have concentrated on the alternative approach of identifying cDNA molecules present on metastatic breast cancer cells by a new panning approach. This corresponds to the task 2 of this grant in the original application as a complement to the peptide sequences identified the previous year through task 1. I will now describe in detail the experimental approach that we have used on the molecules that we have cloned so far.

Establishment of immortalized bone marrow stromal and endothelial cell lines

As described in the previous year, as a complementary approach to in vivo phage display, we decided to include panning in vitro using bone marrow stromal cells, since interaction between bone marrow stromal cells and several types of bone metastatic cancer cells (melanomas, prostate cancer, etc) have been reported. Since it is not known which cell type may play an important role in the initial settlement of breast cancer cells in bone tissue, we focused on both endothelial and stromal cells as putative candidate cell types. The strategy for the immortalization was already described last year.

The Table shows a summary of all the different cell types that have been isolated and characterized based on their expression of alkaline phosphatase (AP), Von Willebrand factor (VWF), VCAM-1, F4/80 or CD44. The cell lines were designated BMS for bone marrow stromal cell-like or BME for bone marrow endothelial cell-like cell lines.

Immortalized Cell Clones

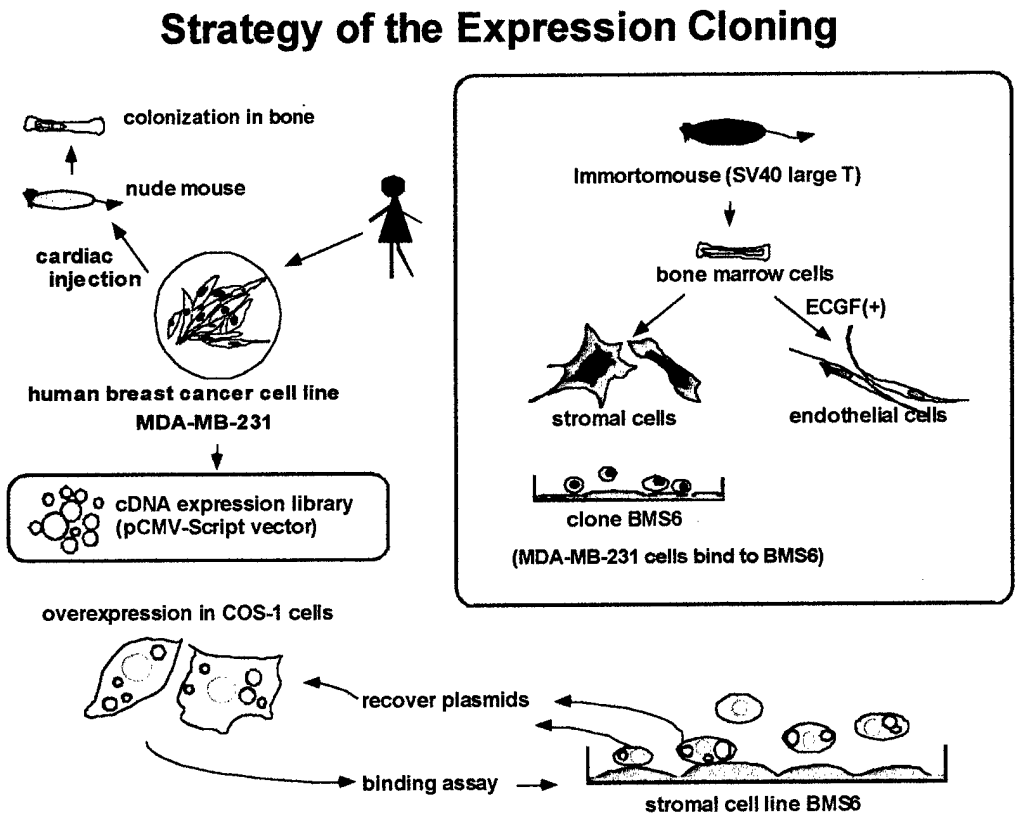
		expression (immunostaining)					binding assay				
	clones	AP	VWF	VCAM -1	F4/80	CD44	MDA-MB -231 (disk)	MDA-MB -231	MDA-MB -361	MCF7	COS-1
stromal cell-like	BMS1	+		-		+++	+	++			
	BMS2	+		-		+	+-	-			
	BMS3	+++		-			++	+++			
	BMS4			-		++					
	BMS5	+++		-		++	+-	+-			
	BMS6	+		-	-	++	+++	+++	-	-	-
	BMS8	+++		-		+	-	+-			
endothelial cell-like	BME1	-					-	++			
	BME2	-					-	++			
	BME3 *		++	++							
	BME4										
	BME5 *	+	++	+++	-		++	+			
	BME6 *	-					-	+			
	BME7						+++	++			
	BME8 *										
	BME9	++					-	++			
	LH23	-						+++			

*If(-)ECGF, looks stromal cell-like

Next we tested the ability of each of these cell lines to bind to the metastatic breast cancer cell line MDA-MB-231 and determine which of the immortalized cell lines would be most useful in developing a panning assay. For this we focused on two different designs of the binding assay.

One is an ordinary binding assay whereby the stromal cell lines or endothelial cell lines are coated on a microtiter plate and the MDA-MB-231 breast cancer metastic cell line is then added to the plates and binding to the bottom of the plate measured with time. The problem with this binding assay is that the unspecific binding of breast cancer cell lines to the stromal and/or endothelial cell lines is high. Therefore we developed a novel assay using a plastic disc that could be coated with the stromal or endothelial cell lines. This disk is then floated on top of medium containing suspended MDA-MB-231 breast cancer cells. This forces the cancer cells to migrate in liquid solution against gravity towards the disc containing the stromal or endothelial cells. We have observed that this binding assay gives very low background. Based on this assay, we chose to use the clone BMS6 as a stromal cell clone since MDA-MB-231 cells bind well in both assay systems and expresses the stromal cell line marker alkaline phosphatase. We also chose BME5 as the endothelial cell clone because of their binding to MDA-MB-231 as well as the expression of endothelial cell markers AP, vWF and VCAM-1. BMS6 or BME5 did not show binding to other types of breast cancer cells such as MCF-7 and MDA-MB-361. MCF7 cells do not colonize in nude mice and MDA-MB-361 are derived from brain metastasis not bone metastasis.

Expression cloning strategy

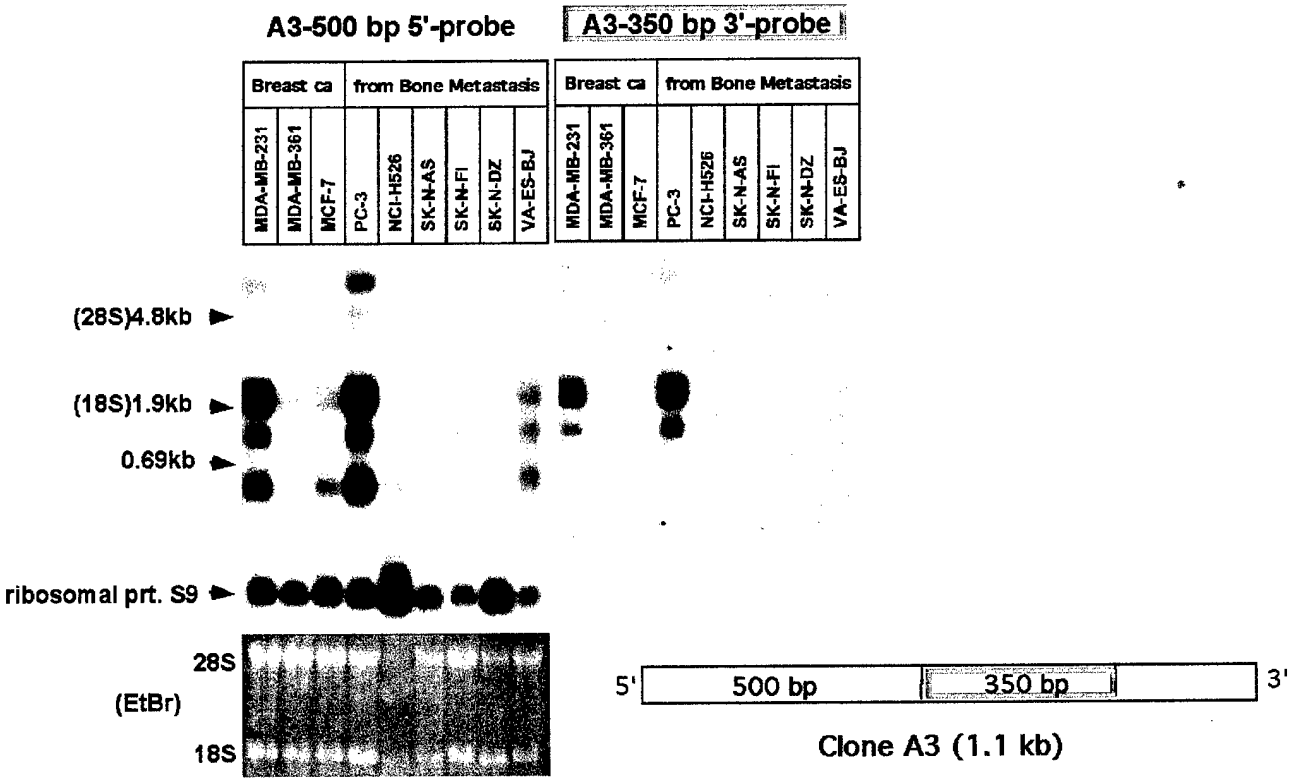


Having these cell lines and panning assays working efficiently, we then focused on developing the expression cloning strategy to try to identify cDNA clones that may mediate the interaction between the MDA-MB-231 metastatic breast cancer cell line and the bone stromal and endothelial cell clones described above

We constructed a cDNA library of MDA-MB-231 cells using a mammalian expression vector, pCMV-Script. This vector carries an SV40 origin of replication, which allows episomal replication in cells that express the SV40 large T antigen, such as Cos-1 cells. We transfected this cDNA library into Cos-1 cells and subjected the transfectants to the binding assay onto the BMS6 cell line. A total of 140 cell clones were isolated and 14 pools of 10 clones each were established and retested in the binding assay. Finally individual all clones were tested.

Two novel cDNA clones have been identified so far with this approach, A3 and A5. We have performed preliminary expression studies using mRNA extracted from the MDA-MB-231 bone metastatic breast cancer cell line, the MDA-MB-361 brain metastatic cells and the MCF-7 non-metastatic breast cancer cell line. We also used other cell lines that are derived from bone metastasis of other cancers. Interestingly clone A3 (shown in Fig. 3) is expressed in MDA-MB-231 cells as well as PC3 cell line, which is a bone metastatic prostate cancer cell line. Using clone A5 on the same mRNAs as before, clone A5 is expressed in most cell lines however it appears higher in breast cancer and prostate cancer but was not discriminating between metastatic and none mestatic cell lines

Northern Blot with Clone A3 (Cancer cell lines)



7. KEY RESEARCH ACCOMPLISHMENTS

- Characterization of bone marrow stromal and endothelial cell lines
- Development of two binding assays to be used in expression cloning by a novel panning procedure
- Cloning and initial characterization of two novel cDNAs mediating the binding of a mestastatic breast cancer cell line to an immortalizedbone marrow stromal cell line.

8. REPORTABLE OUTCOMES

None at this time

9. CONCLUSIONS

We will continue to characterize clone A3 and A5, which appear to be novel molecules. We will generate stable transfectants of these cDNAs into syngeneic mouse tumor cell lines to evaluate whether the cDNA clones are able to target bone tissue in vivo or not and we will expand our analysis of different cell types that may be expressing clone A3 and A5. Prostate cancer cell lines will be of particular interest since it is another tumor type that is also prone to metastasize to bone.